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Accumulation of Non-Superoxide Anion Reactive Oxygen Species Mediates Nitrogen-Limited Alcoholic Fermentation by *Saccharomyces cerevisiae*▽

Ana Mendes-Ferreira,[1] Belém Sampaio-Marques,[2] Catarina Barbosa,[1] Fernando Rodrigues,[2] Vítor Costa,[3,4] Arlete Mendes-Faia,[1] Paula Ludovico,[2] and Cecília Leão[2]*

Institute for Biotechnology and Bioengineering, Centre of Genomics and Biotechnology, Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal[1]; Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal[2]; Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, 4150-180 Porto, Portugal[3]; and Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Departamento de Biologia Molecular, Universidade do Porto, 4099-003 Porto, Portugal[4]

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Throughout alcoholic fermentation, nitrogen depletion is one of the most important environmental stresses that can negatively affect the yeast metabolic activity and ultimately leads to fermentation arrest. Thus, the identification of the underlying effects and biomarkers of nitrogen limitation is valuable for controlling, and therefore optimizing, alcoholic fermentation. In this study, reactive oxygen species (ROS), plasma membrane integrity, and cell cycle were evaluated in a wine strain of *Saccharomyces cerevisiae* during alcoholic fermentation in nitrogen-limiting medium under anaerobic conditions. The results indicated that nitrogen limitation leads to an increase in ROS and that the superoxide anion is a minor component of the ROS, but there is increased activity of both Sod2p and Cta1p. Associated with these effects was a decrease in plasma membrane integrity and a persistent cell cycle arrest at G_0/G_1 phases. Moreover, under these conditions it appears that autophagy, evaluated by *ATG8* expression, is induced, suggesting that this mechanism is essential for cell survival but does not prevent the cell cycle arrest observed in slow fermentation. Conversely, nitrogen refeeding allowed cells to reenter cell cycle by decreasing ROS generation and autophagy. Altogether, the results provide new insights on the understanding of wine fermentations under nitrogen-limiting conditions and further indicate that ROS accumulation, evaluated by the MitoTracker Red dye CM-H_2XRos, and plasma membrane integrity could be useful as predictive markers of fermentation problems.

During wine fermentation, yeast cells are exposed to numerous environmental stresses, which could have adverse effects on cell growth and ultimately may lead to fermentation arrest. In recent years, studies of the yeast transcriptome (5, 20, 22, 30, 36) and/or proteome (34, 38) contributed to a greater understanding of how wine yeasts cope with the various stressful conditions that arise during winemaking. In general, these studies have shown that entrance into stationary phase is the landmark event during wine fermentation, resulting in major transcriptional changes characterized by a general stress response (22). Reactive oxygen species (ROS), including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^\bullet$), are highly toxic oxidants frequently generated by cells in a wide variety of metabolic pathways during normal metabolism. Although ROS have not been considered key determinants of alcoholic fermentations, there is evidence that ROS production is associated with fermentation (12, 17, 31) and that the production of ROS may also be part of the yeast cell response to a variety of stressors that occurs during must fermentations (26). In this context, ROS accumulation may cause oxidative damage to proteins, nucleic acids and lipids, and other cellular components (35), eventually leading to yeast cell death.

Recently, a genome-wide expression study showed that yeast cells respond to the challenge of nitrogen limitation by inducing a great number of genes of respiratory metabolism, for example, those associated with the tricarboxylic acid cycle, oxidative phosphorylation, and oxidative stress response, despite the large amounts of glucose present in the medium (22). In addition, the upregulation of genes involved in cell budding and DNA synthesis is indicative of yeast cell proliferation and suggests that the yeast population is in an unsynchronized state. As found for the majority of eukaryotes, in budding yeast cells the decision to commit to another division cycle, enter a quiescent state, or enter into meiosis takes place during the G_1 phase of the cell cycle (16). Yeast cells resume proliferation when nutritional conditions are favorable but prepare themselves to enter into a resting phase (G_0) under conditions where essential nutrients start to become limiting (37). On the other hand, oxygen availability is known to affect cellular and fermentation processes (28, 29).

The aim of the current study was to obtain new insights on the responsiveness of yeast cells under nitrogen-limiting conditions and specifically to evaluate potential biomarkers that could be used to predict fermentation problems and to optimize fermentation during alcoholic beverage production. To

* Corresponding author. Mailing address: Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal. Phone: 35 1253604818. Fax: 35 1253604809. E-mail: cleao@ecsaude.uminho.pt.
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achieve these aims, the following parameters were evaluated under anaerobic conditions in a wine strain of *Saccharomyces cerevisiae* growing in a nitrogen-limited medium mimicking enological conditions: ROS production, plasma membrane integrity, cell cycle profile, and autophagy induction. The effects of nitrogen addition to a nitrogen-limiting fermentation were also evaluated.

MATERIALS AND METHODS

Strain and maintenance medium. The wine yeast strain *S. cerevisiae* PYCC4072 was supplied by the Portuguese Yeast Culture Collection (PYCC) and maintained at 4°C on slants of yeast peptone dextrose agar (YPD) medium containing glucose (20 g liter^{-1}), peptone (10 g liter^{-1}), yeast extract (5 g liter^{-1}), and agar (20 g liter^{-1}). Fresh cells grown on YPD slants for 24 h were used in all experiments.

Inocula and fermentation conditions. The experimental design was based upon the conditions previously described to ensure continuity in the studies (22) with some modifications. Yeast strain *S. cerevisiae* PYCC4072 was grown in chemically defined grape juice medium (GJM) formulated by Henschke and Jiranek (14). Glucose (200 g liter^{-1}) was used as the sole carbon and energy source, and nitrogen was supplied as diammonium phosphate. Briefly, three fermentation conditions with 200 g liter^{-1} of glucose and different initial nitrogen concentrations were carried out: (i) with 267 mg of N liter^{-1} (GJM_{267}), the nitrogen concentration required by the yeast strain to complete fermentation (control fermentation); (ii) with 67 mg N liter^{-1} (GJM_{67}), the nitrogen concentration that leads to slow fermentation (nitrogen-limiting fermentation); and (iii) with the addition of 200 mg of N liter^{-1} onto the nitrogen-limiting fermentation 72 h after inoculation (GJM_{67+200}), coinciding with 48 h of nitrogen depletion (refed fermentation).

For all experiments starter cultures were prepared by growing the yeast cells overnight in 250-ml flasks containing 100 ml of GJM_{267}. The flasks were incubated at 25°C in an orbital shaker set at 150 rpm. The experimental cultures were inoculated with 5×10^5 CFU ml^{-1} of starter culture. Fermentation was carried out in 500-ml flasks filled to two-thirds of their volume, fitted with a side-arm port sealed with a rubber septum for anaerobic sampling, and maintained at 20°C in an orbital shaker at 120 rpm, according to the methodology in use in our laboratory and described elsewhere (21). Aseptic sampling was accomplished using a syringe-type system. To avoid medium accumulation in the system, a stylet was inserted in the needle holder. Each flask was closed with a rubber stopper, allowing fermentation gasses to escape through a glass tube connected to a two-way valve by Teflon tubing; the other end of the tubing was connected to a tube with 10 ml of distilled water. Every 24 h, each flask was removed from the shaker and weighed. Samples were collected daily for assessing fermentation and growth parameters. Prior to sampling, the flasks were stirred to ensure homogeneity. The end of alcoholic fermentation was confirmed using Clinitest tablets (Roche).

Determination of different growth and fermentation parameters. Optical density at 640 nm (OD_{640}) of appropriately diluted culture samples was used as an estimate of yeast cell growth. The number of viable cells was periodically determined using serial dilutions of the samples which were pour-plated in YPD medium. The number of CFU was counted after 48 h at 30°C. Loss of carbon dioxide was measured by weight loss. Glucose was quantified by the 2,4-dinitrosalicylic acid (DNS) method (25).

Assessment of plasma membrane integrity. Plasma membrane integrity was assessed by flow cytometry using propidium iodide (PI) (Molecular Probes, Eugene, OR) vital staining as described elsewhere (8) with minor adaptations (3). Briefly, PI was added to yeast cell suspensions (10^6 cells ml^{-1}) from a working solution (0.5 mg of PI in 10 ml of Tris-$MgCl_2$ buffer) to a final concentration of 6.7 mg ml^{-1} and incubated at 37°C for 15 min. Cells with high red fluorescence were considered to have plasma membrane disruption.

Assessment of intracellular reactive oxygen species. Intracellular reactive oxygen species accumulation was monitored with the MitoTracker Red dye CM-H_2XRos (Molecular Probes, Eugene, OR) essentially as described elsewhere (18) and with dihydroethidium (DHE) as a probe to detect superoxide anion. The reduced form of this dye does not fluoresce until it enters an actively respiring cell, where it is oxidized by ROS to a red fluorescent compound, which is sequestered in the mitochondria. Briefly, DHE was added to yeast cell suspensions (10^6 cells ml^{-1}) from a working solution (2.5 mg of DHE in 1 ml of absolute ethanol) to a final concentration of 10 μg ml^{-1} and incubated at 30°C for 10 min in dark. The fluorescence was detected by flow cytometry.

Assessment of mitochondrial membrane potential. Mitochondrial membrane potential was monitored by staining cells with the fluorescent dye rhodamine 123 (Rh123) essentially as described elsewhere (19). Rh123 is a cationic dye that distributes electrophoretically into the mitochondrial matrix in response to mitochondrial membrane potential.

Cell cycle analysis. Cell cycle analysis was performed in nitrogen-starved and nonstarved cells according to a previously described procedure (2). Briefly, at the desired time points, cells were harvested, washed, and fixed with ethanol (70%, vol/vol) for 30 min at 4°C, and this was followed by sonication, treatment with RNase for 1 h at 50°C in sodium citrate buffer (50 mM sodium citrate, pH 7.5), and subsequent incubation with proteinase K (1 mg per 10^6 cells). Cell DNA was then stained overnight with SYBR green (10,000×; Molecular Probes), diluted 10-fold in Tris-EDTA (pH 8.0), and incubated overnight at 4°C. Before cytometric analysis, samples were diluted 1:4 in sodium citrate buffer. Determination of cells in each phase of the cell cycle was performed offline with Cylchred and WINMDI software.

Flow cytometry analysis. Fluorescence-activated cell sorter (FACS) analysis was carried out with an Epics XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA) equipped with an argon ion laser emitting a 488-nm beam at 15 mW. The green and red fluorescence were collected through a 488-nm blocking filter. A 550-nm/long-pass dichroic mirror with a 525-nm/band-pass filter and a 590-nm/long-pass with a 620-nm/band-pass filter were used to detect green fluorescence and red fluorescence, respectively. An acquisition protocol was defined to measure forward scatter (FS log), side scatter (SS log), green fluorescence (FL1 log), and red fluorescence (FL3 log) on a 4-decade logarithmic scale. Data (20,000 cells per sample) were analyzed with the Multigraph software included in the System II acquisition software for the Epics XL/XL-MCL, version 1.0.

Protein extract preparation and superoxide dismutase (SOD) and catalase (CAT) activity assays. For determination of catalase and superoxide dismutase activities, yeast extracts were prepared in 25 mM Tris buffer (pH 7.4) containing a cocktail of protease inhibitors. Protein content of cellular extracts was estimated by the method of Lowry using bovine serum albumin as a standard.

Catalase activity was determined as previously described (1). Briefly, 30 μg of protein was separated by native PAGE, and catalase activity was analyzed *in situ* in the presence of 3,3′-diaminobenzidine tetrahydrochloride (Sigma), using the H_2O_2-peroxidase system. The gel was incubated in horseradish peroxidase (50 μg ml^{-1}; Sigma) in 50 mM potassium phosphate buffer (pH 6.7) for 45 min. H_2O_2 was then added to a final concentration of 5 mM, and incubation was continued for 10 min. The gel was then rapidly rinsed twice with distilled water and incubated in 0.5 mg ml^{-1} diaminobenzidine in 50 mM potassium phosphate buffer until staining was complete.

Superoxide dismutase activities were measured based on their ability to inhibit reduction of nitroblue tetrazolium to formazan in nondenaturing polyacrylamide gels (9). Sod2p activity was distinguished from Sod1p activity based on the ability of 2 mM cyanide to inhibit Sod1p but not Sod2p. Quantification of band intensities was performed by densitometry using Quantity One Basic Software from Bio-Rad.

RNA analysis. RNA was obtained from samples periodically collected in the slow (GJM_{67}) and control (GJM_{267}) fermentations as well as in refed fermentation (GJM_{67+200}). RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) standard procedures and heat shock treatment (20 min at 65°C followed by 60 min at −80°C) for cellular disruption. Total RNA (250 ng) was reverse transcribed using a SuperScript III Platinum Two-Step qRT-PCR kit with SYBR green from Invitrogen. One microliter of the reverse-transcribed RNA was used as a template to amplify the genes, using primers to the *ATG8* gene (sense, 5′-GAAGGCGGAGTCGGAGAGG-3′; antisense, 5′-GGCAGACATCAACG CCGC-3′) and to the *ACT1* gene (sense, 5′-CACCAACTGGGACGATATGG-3′; antisense, 5′-GAAGGCTGGAACGTTGAAAG-3′). The expression of the *ATG8* gene was assessed by real-time reverse transcription-PCR (RT-PCR) in a DNA Engine Opticon CFX98 C1000 Thermocycler (Bio-Rad). Results obtained were normalized to the reference gene *ACT1*. The data obtained were analyzed by applying the Livak method or the $2^{-\Delta\Delta CT}$ method (32) in order to verify the effect of nitrogen on expression levels, as follows: $\Delta\Delta C_T = (C_{T(\text{target gene})} - C_{T(\text{reference gene})})\text{test} - (C_{T(\text{target gene})} - C_{T(\text{reference gene})})\text{calibrator}$, where C_T is threshold cycle.

Statistical analysis. Data are reported as mean values of at least three independent assays and presented as means ± standard deviations (SD). The arithmetic means are given with standard deviations with 95% confidence value. Statistical analyses were carried out using a Student's *t* test or two-way analysis of variance (ANOVA). *P* values of less than 0.05 were considered statistically significant and are marked at the tops of the columns or the curve points in the figures.

RESULTS AND DISCUSSION

Anaerobic fermentation profiles under different nitrogen conditions. During wine fermentation, yeast cells are exposed to environmental stresses such as nitrogen depletion that can negatively affect cell growth and cell viability and ultimately lead to fermentation arrest. In the present work, the effects of nitrogen limitation during fermentation were mimicked using the wine yeast strain *S. cerevisiae* PYCC4072 in a grape must synthetic medium with high and low nitrogen contents under anaerobiosis. Nitrogen depletion effects on cell density, viability, and fermentation activity were assessed (Fig. 1). Nitrogen limitation (GJM_{67}) resulted in lower fermentative activity, as reflected by the high glucose concentrations and low CO_2 levels present in the medium at 168 h compared to the control fermentation (GJM_{267}), where glucose was completely fermented (Fig. 1C and D). Consistently, CFU analysis showed that under nitrogen limitation conditions (GJM_{67}), the number of CFU from 48 h on is significantly lower than that of the control fermentation, reflecting the early arrest of yeast cell division (Fig. 1A and B). After nitrogen refeeding at 72 h, yeast growth was reinitiated, and fermentation (measured as glucose consumption and CO_2 production) was completed with a delay of 24 to 48 h compared to the control fermentation (Fig. 1). These results are in agreement with the previous characterization of fermentation profiles applying similar nitrogen conditions although under a microaerophilic environment (22, 23).

Different physiological parameters were then assessed with the aim of gaining new insights on the mechanisms underlying the low fermentative activity observed under the above described nitrogen conditions.

Nitrogen depletion induces ROS accumulation. Intracellular ROS accumulation has been described as the result of stress promoted by different nutritional and environmental factors that affect grape must fermentation (4, 12, 17, 26). To further characterize the mechanisms underlying the slow fermentation observed under the above described nitrogen-limiting conditions (Fig. 1), the levels of intracellular ROS were periodically evaluated by flow cytometry using dihydroethidium (DHE) and MitoTracker Red CM-H_2XRos (MitoTracker) as probes, respectively, sensitive to superoxide anion and overall cellular ROS (Fig. 2). According to data previously reported for hypoxic fermentation in high glucose-containing medium (17), it was observed that ROS are accumulated in 10 to 20% of cells undergoing the control fermentation, as revealed by both probes (MitoTracker and DHE) (Fig. 2). Nitrogen depletion resulted in an increased percentage of cells with ROS accumulation (40 to 50%) when ROS were evaluated by MitoTracker Red CM-H_2XRos (Fig. 2A) but not by DHE (Fig. 2B), excluding the time point 24 h, when there are no major differences between the control and N-starved fermentations in what concerns the fermentation profiles, and at 72 h, when nitrogen depletion seriously compromised growth (Fig. 1). These results, although indicating that nitrogen depletion led to ROS accumulation, suggest that superoxide anion was a minor contributor to the overall ROS as determined by MitoTracker Red CM-H_2XRos. Nitrogen refeeding at 72 h resulted in a significant decrease in the percentage of cells with high ROS content compared to nitrogen-limiting conditions; the final ROS levels in refed cells were in fact much lower than

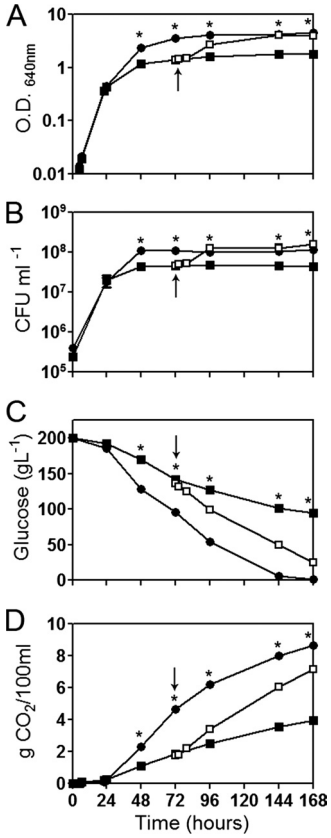

FIG. 1. Anaerobic fermentation profiles under different nitrogen conditions. Cell density, viability, and fermentation rate of *S. cerevisiae* PYCC4072 grown in synthetic grape juice medium at 20°C under different nitrogen regimes were measured: cell density evaluated by turbidimetry (OD_{640}) (A); cell viability determined by counting CFU (B); concentration of glucose and CO_2, (C and D, respectively). Three main fermentation conditions with 200 g liter^{-1} of glucose were established by manipulating the initial nitrogen concentration in the culture medium: ●, 267 mg of N liter^{-1}, the nitrogen concentration required by the yeast strain to complete alcoholic fermentation (control fermentation); ■, 67 mg of N liter^{-1}, the nitrogen concentration of the N-limiting fermentation; and □, the addition of 200 mg of N liter^{-1} into the N-limiting fermentation, 72 h after inoculation (indicated by arrow), coinciding with 48 h of nitrogen depletion (refed fermentation). Values are presented as mean ± standard deviation from three independent experiments. Statistical significance (*, $P < 0.05$, between the control and N-starved fermentations) was determined by two-way ANOVA.

those found in the control fermentation at the later time points (Fig. 2). Similar to results under nitrogen-limiting conditions, superoxide anion was not a major contributor to the overall ROS in nitrogen-refed fermentation (Fig. 2). To evaluate the relevance of mitochondrial membrane potential alterations on the generation of superoxide anions, cells were stained with rhodamine 123 (Rh123). The current results showed that nitrogen-limiting conditions lead to a decrease of mitochondrial membrane potential throughout fermentation, as represented for 96 h (Fig. 2C). Thus, under nitrogen-limiting conditions, it can be hypothesized that lower mitochondrial membrane potential could account for the lower levels of superoxide anions detected. However, it is also necessary to consider that superoxide dismutase (SOD) activities are increased as well (Fig. 3).

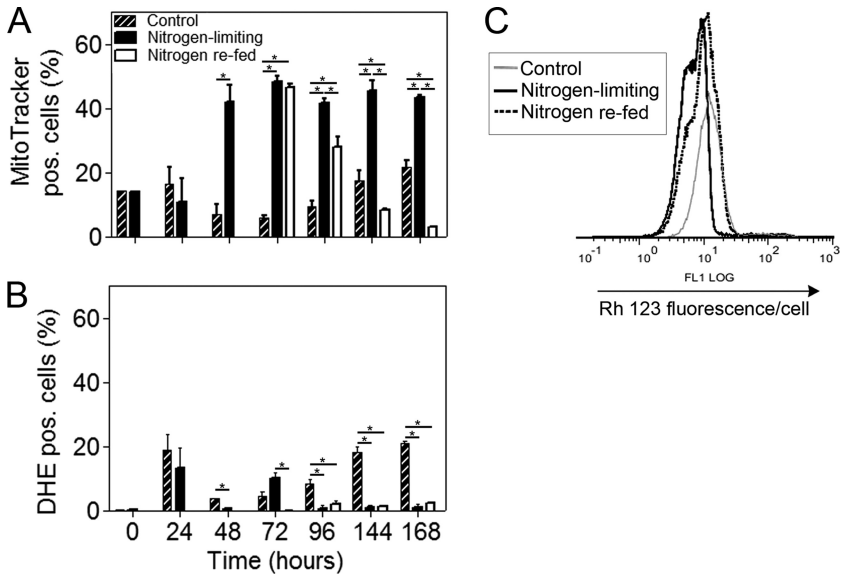

FIG. 2. Nitrogen depletion induces ROS accumulation associated with a lower mitochondrial membrane potential. FACS measurements of intracellular ROS using MitoTracker Red CM-H_2XRos (MitoTracker) (A) and of superoxide anions using the superoxide-specific probe DHE (B) in *S. cerevisiae* cells under different fermentation conditions. Bar graphs indicate the percentage of cells exhibiting high levels of intracellular ROS detected by FACS measurements (20,000 cells per sample in three independent experiments) of MitoTracker- and DHE-positive cells. Values are mean ± standard deviations of all experiments. Statistical significance (*, $P < 0.05$) was determined by a Student's *t* test. (C) Overlay of FACS green fluorescence histograms (FL1 log) representative of *S. cerevisiae* cells obtained at time 96 h from the different fermentations and stained with rhodamine 123 (Rh123) as an indicator of mitochondrial membrane potential.

Sod2p and Cta1p activities are increased under nitrogen-limiting fermentations. The cellular responses to ROS include the induction of enzymatic antioxidant defense systems such as catalase (CAT) and superoxide dismutase to maintain physiological homeostasis. To evaluate the role of antioxidant systems on ROS accumulation promoted by nitrogen-limiting conditions, activity measurements were made for superoxide dismutases (Sod1p, which is Cu/Zn dependent, and Sod2p, which is mitochondrial and Mn-dependent) and catalases (Cta1p, which is peroxisomal, and Ctt1p, which is cytosolic), both classes of enzymes playing essential roles in protecting yeast cells against oxidative stress (15). Globally, cells from nitrogen-limiting conditions displayed Sod1p, Sod2p, and Cta1p activities higher than those observed in the control fermentation (Fig. 3). Nevertheless, Sod2p and Cta1p displayed an increased activity over time compared to Sod1p activity (Fig. 3). These results are in accordance with previous reports showing that ROS, particularly sublethal H_2O_2 concentrations, induce the transcription of *SOD1* and *SOD2* genes but with a more robust induction of Sod2p activity (11, 13, 24). Moreover, the increased Sod2p activity is most probably responsible for the lower levels of superoxide anion and their irrelevant contribution to overall ROS observed under N-starved fermentation. Nitrogen refeeding resulted in a global kinetic enzymatic activity pattern similar to that found for nitrogen-limiting fermentation (Fig. 3) with a slight decrease over time, probably reflecting the observed cell growth (Fig. 1). Consistent with anaerobic conditions and the regulation of Ctt1p by oxygen (6), no activity of this enzyme was detected at any of the time points analyzed.

Overall, nitrogen-limiting fermentations under either anaerobic (Fig. 3) or microaerophilic conditions (from previous *in silico* data of the genome-wide expression analysis in *S. cerevisiae* PYCC4072 [22]; accession number GSE5842 [www.ncbi.nlm.nih.gov/geo/]) suggest that the antioxidant enzymatic defense systems, superoxide dismutase and catalase, are triggered to counteract the increased ROS generation (Fig. 2). The current findings further indicate a major role of Sod2p and Cta1p in nitrogen limitation under anaerobic conditions.

Nitrogen limitation induces cell membrane damage and cell cycle arrest in G_0/G_1 phases correlated to ROS accumulation. The results presented above demonstrate that in spite of the increased Sod2p and Cta1p activities, nitrogen limitation affects fermentation performance by causing increased ROS accumulation. To evaluate whether ROS accumulation was accompanied by damage to cell structures, the ability of plasma membrane to effectively exclude propidium iodide (PI) was assessed by flow cytometry. Plasma membrane integrity of cells from the control fermentation was mainly unaffected while under nitrogen-limiting conditions the percentage of PI-positive cells significantly increased from early time points (Fig. 4). After nitrogen refeeding, the number of cells with loss of plasma membrane integrity was significantly decreased (Fig. 4). Altogether, the results showed that nitrogen-limiting conditions induce loss of plasma membrane integrity, which is directly correlated with increased ROS accumulation and with lower survival than in the control and refed fermentations (Fig. 1 and 4), promoting the link between the two phenomena, ROS accumulation and plasma membrane injury.

To further understand the impact of ROS accumulation induced by nitrogen-limiting conditions, cell cycle progression was analyzed. In comparison to the control fermentation, nitrogen-limiting conditions promoted a persistent cell cycle arrest in phases G_0/G_1 at 48 h when nitrogen was completely

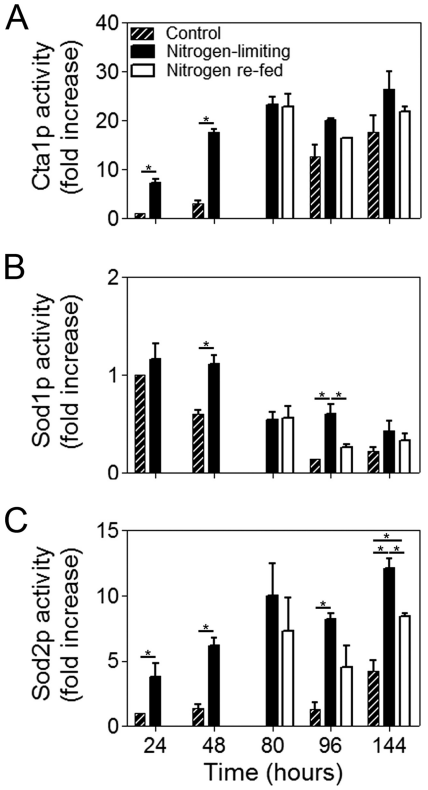

FIG. 3. Sod2p and Cta1p activities are increased under nitrogen-limiting fermentations. Quantification of fold increases in the activity of peroxisomal catalase (Cta1p) (A), Cu/Zn-dependent superoxide dismutase (Sod1p) (B), and mitochondrial Mn-dependent superoxide dismutase (Sod2p) (C) of *S. cerevisiae* cells over time under the different indicated fermentation conditions. Cta1p, Sod1p, and Sod2p activity at each time point was normalized to the activity of cells from the control fermentation at time 24 h. Values are presented as mean ± standard deviation from three independent experiments. Statistical significance (*, $P < 0.05$) was determined by a Student's *t* test.

exhausted from the medium (22, 23) (Fig. 5B). Nitrogen depletion-mediated G_0/G_1 cell cycle arrest is a well-known phenomenon that seems to be modulated by downregulation of the G_1 cyclin *CLN3* (10, 22). Nitrogen refeeding at 72 h allowed cells to reenter cell cycle (Fig. 5C), as reflected by cell density and viability (Fig. 1) and reduction of ROS accumulation (Fig. 2).

Overall the results suggest a close relationship between increased ROS accumulation and negative effects on cell membrane integrity and cell cycle. Both results obtained under nitrogen-limiting conditions and also after nitrogen refeeding support this hypothesis.

Autophagy triggered by nitrogen limitation is not sufficient to avoid slow fermentation. Autophagy is known as a multifunctional mechanism associated with different physiological and cellular processes in eukaryotes. A key regulatory component of autophagy is Atg8p, a ubiquitin-like protein that acts in the vesicle enlargement step, playing an important role in the cross talk between autophagy and other cellular and physiological networks (7, 27). To assess whether the yeast cells could respond to nitrogen limitation by inducing autophagy, mRNA

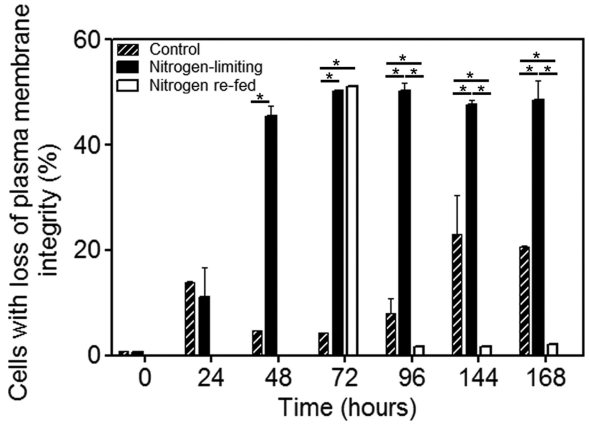

FIG. 4. Nitrogen limitation induces cell membrane damage. Percentage of *S. cerevisiae* cells displaying affected plasma membrane integrity evaluated under the three different nitrogen regimes, as assessed by FACS quantification of cells after vital staining with propidium iodide.

levels of the widely known marker gene *ATG8* were monitored by RT-PCR in yeast cells collected under the different fermentation conditions (Fig. 6).

ATG8 gene expression was observed at relatively low levels

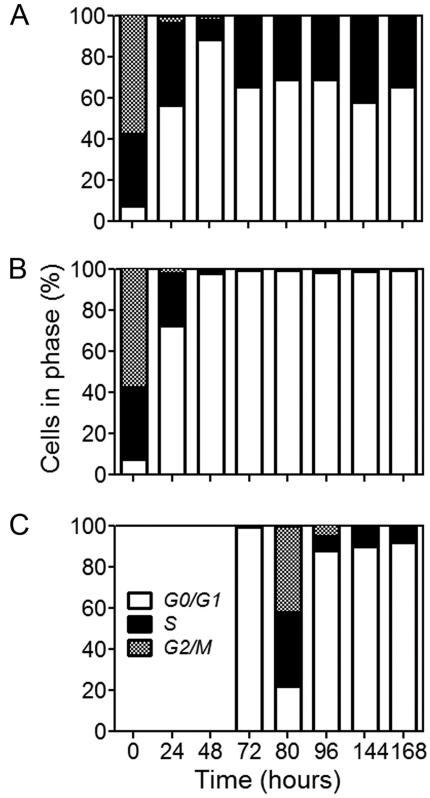

FIG. 5. Nitrogen limitation induces cell cycle arrest in G_0/G_1 phases. FACS analysis of the percentage of cells in each phase of the cell cycle using SYBR green staining of *S. cerevisiae* to measure DNA content. (A) Control fermentation. (B) Nitrogen-limiting fermentation. (C) Nitrogen refeeding after 72 h. Bar graphs correspond to a representative experiment performed out of three independent experiments.

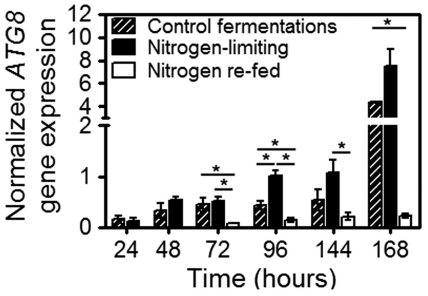

FIG. 6. Nitrogen limitation induces increased expression of *ATG8*, a marker of autophagy. Normalized fold expression levels of the *ATG8* gene (actin, *ACT1*, was used as internal reference), evaluated by RT-PCR, in *S. cerevisiae* cells under the three different nitrogen regimes. Values indicate mean ± standard deviation from three independent experiments. Statistical significance (*, $P < 0.05$) was determined by a Student's *t* test.

and increased during the time course under all the fermentation conditions, reflecting either carbon or nitrogen limitation (Fig. 6). Nonetheless, the highest expression levels of the *ATG8* gene were observed under nitrogen-limiting conditions at 168 h (Fig. 6), corroborating previous studies where autophagy is essential to allow yeast survival in the absence of nitrogen (33). Furthermore, these data also support the theory that autophagy triggered by nitrogen limitation is not sufficient to evade slow fermentation (Fig. 1). Conversely, nitrogen refeeding induced a decrease in the mRNA levels of the *ATG8* gene throughout fermentation, indicating that the nitrogen supply overcomes cellular needs of the autophagic process. Overall, and of utmost relevance, triggering autophagy under nitrogen-limiting fermentation is not sufficient to prevent low fermentation activity.

Our previous study revealed that nitrogen limitation induces several different transcriptional alterations compatible with a yeast general stress response (22). The data presented herein obtained with a complementary physiological approach is the natural next step that gives further insights on the cellular effects of nitrogen limitation under conditions mimicking wine fermentation. Of relevance, nitrogen limitation triggers ROS accumulation, without involvement of the superoxide anion, and this is associated with increased activity of some antioxidant enzymes, particularly, Sod2p. These phenomena are associated with increased loss of plasma membrane integrity, cell cycle arrest at G_0/G_1 phases, and induction of autophagy. Importantly, the induction of autophagy under nitrogen-limiting conditions is not sufficient to provide an identical control fermentation profile. In fact, fermentation reestablishment was observed only when nitrogen was added into the nitrogen-limiting fermentation, and the overall negative cellular effects were completely reverted. From an applied point of view, the present work further emphasizes the relevance of nitrogen availability during grape must fermentation and reveals that both ROS accumulation, measured by MitoTracker, and loss of plasma membrane integrity, but not *ATG8* expression, could be useful as markers for early detection of nitrogen limitation in wine fermentations.

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